The Effect of Undernutrition in the Development of Frailty in Older Persons

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Pituitary Alterations Involved in the Decline of Growth Hormone Gene Expression in the Pituitary of Aging Rats

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Growth hormone (GH) declines during aging. This study investigates whether pituitary constitutive alterations may be involved in the GH decline. Two groups of male Wistar rats were studied (young: 3-month-old; old: 24-month-old). The old rats showed lower pituitary GH messenger RNA (mRNA) levels, immunoreactive rat (IR)-GH content, and GH secretion with no difference in pituitary Pit-1 and cAMP-response element-binding protein (CREB) expression. Pituitary GH releasing hormone receptor (GHRH-R), GH secretagogue receptor (GHS-R), sstr2, and sstr5 mRNA levels were significantly reduced in old rats. The percentage of GH immunoreactive cells was similar in both groups. In vitro, pituitary IR-GH response to GHRH, forskolin (FK), ghrelin, and insulin-like growth factor I (IGF-I) was similar when compared with respective basal secretion and somatostatin-diminished GHRH- and ghrelin-induced IR-GH release in both groups. These results indicate that, as somatotrope function is maintained in aging, the changes observed in GH gene expression and secretion could be reversed by GHS.

IT is now evident that the joint decline in growth hormone (GH) secretion (1,2) rate and plasma insulin-like growth factor I (IGF-I) concentrations (3,4) is one of the best characterized events that occurs with aging (5). GH secretion in male rats is pulsatile in nature (6), and in old male rats the amplitude and duration of the pulses decrease, but the periodicity appears to be similar to that present in younger rats (7).

Previous studies from our laboratory indicated that alterations in GH gene expression might account for the decrease of GH secretion in aging (8). These studies also demonstrated that the decrease of GH gene expression and secretion are due to aging and not to the increase in body weight that occurs with aging. We also confirmed that aging is not a physiological situation of GH resistance, and that the peripheral alterations of the GH–IGF-I system are reversible by repetitive exogenous GH administration (9).

GH is physiologically regulated by the opposite actions of at least two specific neuropeptides, growth hormone releasing hormone (GHRH) and somatostatin (SS) (10). GHRH stimulates GH secretion and GH gene expression, whereas SS inhibits GH secretion through the activation of their specific receptors: GHRH receptor (GHRH-R) and SS receptors (11,12). It has recently been reported that ghrelin, a potent acylated peptide synthesized in the stomach, anterior pituitary gland, and hypothalamus, drives GH secretion via a novel cascade of hypothalamo-hypophyseal mechanisms with the participation of the GH secretagogue receptor (GHS-R) (13–16). Five different SS receptor subtypes (sstr1–sstr5) have recently been cloned and characterized in various species, including the human, rat, and mouse (17,18). Sstr2 and sstr5 are the most abundant and functionally active SS receptor subtypes in the rat pituitary, where they mediate the suppressive action of SS on GH secretion (19,20). Binding of SS to its receptor inhibits the signal transduction systems for both GHRH and GHS, and produces a reduction of basal and stimulated GH secretion (21,22). Pituitary expression of the GH gene is dependent on a pituitary-specific transcription factor-1 (Pit-1) (23). Pit-1 transcription is regulated by the cAMP-response element-binding protein (CREB), a positive autoregulation by its own gene product, and by other pituitary-specific transcription factors (24–26). In addition, it has been reported that CREB plays a role in pituitary GH production and somatotrope expansion (27).

It is not known why GH secretion declines with increasing age. Possible mechanisms, alone or in combination, include reduced GHRH secretion or action, increased SS secretion or action, and reduced somatotrope number or function. It has been suggested that changes in the specific hypothalamic regulatory hormones might be the primary etiological factor (28), but primary pituitary alterations should also be considered because early studies suggested that it is the pituitary itself that undergoes senescent damage (29). It is known that somatotrope responsiveness to the specific regulatory neuropeptides undergoes wide variations during the rat life span. Somatotrope responsiveness to GHRH gradually decreases with age, although there is no general consensus on this point (30,31). Some studies have shown a diminished capacity of the secretagogues to release GH in vivo with age (32), and a higher sensitivity to SS with aging has also been postulated (33).

Circulating IGF-I, a primary target gene of GH action, also participates in the negative feedback regulation of GH

by acting at the hypothalamus and the pituitary levels through an endocrine long loop feedback (34). IGF-I decreases GH secretion and messenger RNA (mRNA) levels in primary rat pituitary cells and human pituitary tumor cell cultures (35,36). It has been postulated that an increase in the sensitivity of the somatotrope to the inhibitory action of IGF-I may be involved in the GH decline with aging (37).

The aim of this study was to elucidate whether the decline in GH gene expression during aging is a constitutive alteration of the pituitary gland due to changes in some of the pituitary transcription factors involved in GH gene expression, such as pituitary Pit-1 and/or CREB; an alteration of pituitary GHRH-R, GHS-R, SS subtypes sstr2 and sstr5 receptors and/or IGF-I-R, or a reduction of somatotrope number or function. The involvement of these age-related pituitary alterations was determined by analyzing the responsiveness of pituitary cells of aging rats to the GH stimulatory factors GHRH and ghrelin and to the GH inhibitory factors SS and IGF-I. The outcome of this study might provide the experimental support for a potential reversibility of these alterations using secretagogues in older humans.

Methods

Animals

Young adult (3-month-old) and old (24-month-old) male Wistar rats were obtained from the Animal Experimental Center (Universidad de Alcalá, Madrid, Spain). Upon arrival at our institution, animals were housed in a specific pathogen-free facility and maintained on a 12-hour light/dark cycle. Water and food were available ad libitum to all animals. The maintenance and handling of the animals were performed as recommended by the National Institutes of Health (NIH) guidelines on the care and use of laboratory animals, according to the principles expressed in the Declaration of Helsinki and with the guidelines on protection of animals used in scientific research set by the Spanish Real Decreto 223/1988 March 14, 1989 and Orden October 13, 1989. For pituitary tissue extract studies, four experiments using three male Wistar rats in each group were performed. For the pituitary cell culture studies, four experiments were done with three to four dishes used per condition. The animals were killed by decapitation, and their pituitary glands were removed under sterile conditions; the neurohypophyses were discarded, and the anterior pituitaries rapidly frozen on dry ice and stored at –80°C until extraction and quantification of the different parameters, or collected and mechanico-enzymatically dispersed for preparation of primary pituitary cell cultures.

Reagents

Rat GHRH (1-43) was purchased from Neo System (Strasbourg, France). Rat ghrelin and SS-14 were obtained from Bachem (Torrance, CA). Recombinant human IGF-I was obtained from PeproTech EC (London, U.K.). ^{125}I, [^{32}P]deoxy-CTP and [^{32}P]ribo-UTP were obtained from Amersham Pharmacia Biotech (Amersham, Buckinghamshire, U.K.). Poly-L-ornithine was obtained from Sigma (St. Louis, MO).

Buffers and Media

Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), Hank's balanced salt solution (HBSS), phosphate-buffered saline (PBS), penicillin–streptomycin, and L-glutamine were obtained from BioWhittaker (Walkersville, MD). Defined medium consisted of DMEM (glucose 1 g/L) supplemented with: bovine serum albumin (BSA) 1%, HEPES (15 mM), hydrocortisone (0.1 μM), T3 (0.5 nM), transferrin (10 μM), glucagon (10 nM), and parathyroid hormone (PTH) (0.2 nM) (obtained from Sigma) and L-glutamine (4 mM) and penicillin–streptomycin (100 U/mL).

Cell Cultures and Experimental Design

Pituitary cells from the two groups of rats were cultured as previously described (38). Anterior pituitaries of 3-month-old and 24-month-old male Wistar rats were collected and mechanico-enzymatically dispersed with 0.1% papain, 0.1% neutral protease, and 0.1% DNase for 1 hour at 37°C. The dispersed pituitary cells were resuspended in defined medium containing FCS (5%), plated on poly-L-ornithine-coated culture plates (24-well) and seeded at a density of 4×10^5 cells per dish. Cultures were kept in a humidified atmosphere of 5% CO_2/95% air at 37°C. After a stabilizing period of 72 hours in serum-supplemented defined medium (5% FCS), cells were preincubated in defined medium (serum free) for 4 hours and treated with GHRH, forskolin (FK), ghrelin and/or SS-14 for another 4 hours, and with recombinant human IGF-I for another 72 hours. At the end of the experiments, media were removed, and cells acidified with 0.1 M hydrochloric acid ClH; both were boiled for 5 minutes and centrifuged for 30 minutes at 20,000 *g*. The supernatants were immediately frozen and stored at –20°C until being analyzed for GH concentration by radioimmunoassay (RIA).

Cell Culture Validation

To determine the behavior of the somatotropes from young and old rats in our in vitro system, anterior pituitaries of 3-month-old and 24-month-old male Wistar rats were plated on six-well culture plates and seeded at a density of 1×10^6 cells per dish as previously described. After the stabilizing period, media were replaced and the cells were incubated for 72 hours. To test for IR-GH release, aliquots of media were taken after 4, 8, 24, 48, and 72 hours. To test for GH mRNA levels, cells were obtained after 4 or 72 hours, washed with PBS, and removed in TRIzol reagent (Life Technologies, Inc., Grand Island, NY) for total mRNA extraction and Northern blot analysis as described below. Preliminary studies demonstrated that GH release increased with increasing doses of GHRH, GHS, and FK and decreased with increasing doses of SS or IGF-I. Time-course studies showed that these effects were more evident 4 hours after GHRH, GHS, FK, and SS and 72 hours after IGF-I addition.

Table 1. Primers Used in Polymerase Chain Reaction

Target Gene	Gene Bank Accession	Forward Primer (5′ → 3′)	Reverse Primer (5′ → 3′)	Length (bp)
GHS-Rα	U94321	AGGCAACCTGCTCACTATGCTG	GACAAGGATGACCAGCTTCAGC	292
sstr2	NM_019348	ATGTCTCTGCCGCTCTTG	GCCAGGTTGACGATGTTG	217
sstr5	NM_012882	GCAGCCTTCATCACCTAC	ACAAACACCAGCACCAC	317
GAPDH	NM_017008	ACCCATCACCATCTTCC	GGTTCACACCCATCACA	194

Note: GHS-Rα = GH secretagogue receptor alpha.

GH RIA

Immunoreactive rat GH (IR-GH) in pituitary extracts and culture media was measured by RIA using the National Pituitary Hormone Distribution Program rat hormone Kit (National Institute of Diabetes and Digestive and Kidney Diseases [NIDDKD], Bethesda, MD) with a sensitivity limit of 0.8 μg/L. IR-GH was measured in individual rats or wells. All samples from each experiment were measured in the same RIA to exclude inter-assay variation, and GH values were expressed as percentages of the 3-month-old control group.

Western Analysis

Pituitaries were lysed in a buffer containing 50 mM NaCl, 0.01 M Tris-HCl (pH 7.6), 0.001 M EDTA, 0.1% Nonidet P-40, aprotinin at 1 μg/mL, and phenylmethylsulfonyl fluoride (PMSF) at 100 μg/mL. Total protein extracts (10 μg) were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane. After blocking the membranes, immunodetection was performed using rabbit anti-Pit-1 (1:5000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), anti-CREB (1:1000; Cell Signaling Technologies, Beverly, MA), anti-P-CREB (Ser^{133}) (1:1000; Upstate Biotechnology, Lake Placid, NY), and anti-IGF-I-Rβ (1:1000; Santa Cruz Biotechnology) followed by incubation with a goat peroxidise-conjugated antirabbit secondary antibody (1:1000 dilution; DAKO, Glostrup, Denmark). Immunoreactive bands were visualized using an enhanced chemiluminescence-detecting system (Amersham). Quantification of the intensities of the autoradiographic bands was done by scanning densitometric analysis of the x-ray films using National Institutes of Health (NIH) Image 1.47 and Adobe-Photoshop 2.0 software (Macintosh). As a loading control, the membranes were systematically stained with Coomassie brilliant blue R-250 solution (0.125%), for 1 hour at room temperature, to validate the Western blot.

Northern Analysis

Total RNA was extracted using TRIzol reagent, according to the protocol supplied by the manufacturer. A fraction of 2–15 μg was subjected to electrophoresis in a 1% agarose/0.66 mol/L formaldehyde gel, followed by electrotransfer to a nylon membrane (Nytran; Schleicher & Schuell, Keene, NH) and ultraviolet cross-linking (Hoefer Scientific Instruments, San Francisco, CA). The complementary DNA (cDNA) probes for GH, GHRH-R, and Pit-1 were labeled with [^{32}P]deoxy-CTP (3000 Ci/mmol), and CREB riboprobe was labeled with [^{32}P]ribo-UTP (3000 Ci/mmol). Prehybridization was carried out for 16 hours at 42°C, and membranes were hybridized for 24 hours at 42°C using UltraHyb (Ambion, Austin, TX) with approximately 1×10^6 cpm/mL labeled antisense GHRH-R, Pit-1, and CREB probe, and 5×10^5 cpm/mL labeled antisense GH probe. Autoradiograms and quantifications of intensity were done as described above. Equal loading was confirmed, and data were expressed as arbitrary units after correction for the 18S RNA.

Ribonuclease Protection Assay

Total RNA was hybridized overnight with approximately 600,000 cpm of labeled antisense rat IGF-I-R at 45°C. The hybridization solution contained 75% (vol/vol) formamide, 80 mM Tris-HCl (pH 7.6), 4 mM EDTA, 1.6 M NaCl, and 0.4% SDS. After hybridization, samples were digested using RNase A (40 μg/mL) and RNase T1 (2 μg/mL) for 1 hour at 30°C. Protected hybrids were isolated by ethanol precipitation after phenol–chloroform extraction and were separated according to size on an 8% polyacrylamide/8 M urea denaturing gel. Gels were exposed to x-ray film (Kodak, Cambridge, U.K.) at –80°C for 24–36 hours. Quantification of the intensities of the autoradiogram bands corresponding to protected hybrids was done by densitometric scanning as described above. All samples were hybridized at the same time with 18S RNA to correct for the differences in gel loading.

Semiquantitative Reverse Transcriptase–Polymerase Chain Reaction

Total RNA isolated from pituitary extracts was used as template for GHS-R, sstr2, sstr5, and GAPDH. Briefly, total RNA (2 μg) was reverse transcribed using random primer and Superscript II reverse transcriptase (RT; Invitrogen, Carlsbad, CA). Sets of specific primers for rat GHS-R, sstr2, sstr5, and GAPDH were designed using LightCycler Probe Design Software 1.0 (Roche Diagnostics, Mannheim, Germany) based on the sequence data of the gene available in GeneBank (Table 1).

The sets of primers were used for LightCycler real time polymerase chain reaction (PCR) using Sybr Green I (Roche Applied Science, Indianapolis, IN). The reaction was performed in a total volume of 20 μL in microcapillary tubes, according to the manufacturer's instructions. Each reaction mixture contained 5 μL of cDNAs, 2 μL of FasterStart DNA Master Sybr Green I mix, 2 μL of sense and antisense primers each (0.5 μM), 2.4 μL of 25 mM $MgCl_2$, and 6.6 μL of PCR-grade H_2O. The LightCycler programs for each gene were as follows: denaturation (95°C/10 minutes); PCR amplification and quantification (95°C/15 seconds, 58–60°C/5 seconds, 72°C/12 seconds) with single fluorescence measurement at the

end of the elongation step, repeated for 40 cycles. The transition temperature at all steps was 20°C/s. To verify that only the specific product was amplified, a melting point analysis was conducted after the final cycle. Specific amplification was also confirmed by electrophoresis of PCR products on 2% agarose gel.

Data acquisition and analysis were performed using LightCycler software 3.5 (Roche). Threshold cycle (C_T) was determined by second derivative maximum analysis. Specific standard curves for each gene were generated by serial dilution (1 to 1/100) of total pituitary cDNAs to determine the lineal amplification range and reaction efficiency. All samples were tested in triplicate. Each run included a nontemplate control to test for contamination of assay reagents. For each sample the value of the housekeeping gene GAPDH was analyzed as a standard for the quality of the RNA samples investigated. No regulation of pituitary GAPDH gene expression by age was confirmed by Northern blot. Relative target mRNA expression from old to young rats was calculated by using the $2^{-\Delta\Delta CT}$ method described by Livak and Schmittgen (39).

Fixation, Staining, and Flow Cytometry of Cells

To assess the potential variation in the number of somatotropes per pituitary with age, the percentage of GH-positive cells was determined using immunofluorescence flow cytometry. Pituitary cells were dispersed by the same procedure used for primary culture, and immunostaining performed using the reagent Fix&Perm Staining Cell Permeabilization Kits (Caltag Laboratories, Burlingame, CA), according to the protocol supplied by the manufacturer. Cells were incubated with sheep antirat GH (1:1500; NIDDKD). Then a second antibody conjugated to fluorescein isothiocyanate (FITC; antisheep immunoglobulin G [IgG]; INC Biomedicals, Aurora, OH) and 7-amino-actinomycin D (7-AAD; Via-Probe; BD PharMingen, Becton Dickinson Co., San Diego, CA) were added. The cells were then analyzed on a FACScan Flow Cytometer (Becton-Dickinson, San José, CA). In parallel, one aliquot was treated with RNAse (Roche) and propidium iodide as described (40) to assure that single nuclear cells were analyzed. A gate was set in a 7-AAD forward scatter (FSC) dot-plot so that only nucleated cells were analyzed. The percentage of somatotropes was determined in an FITC histogram.

Statistical Analysis

All data are expressed as the mean ± standard error (*SE*). Tests for significance between sample groups were performed with a two-tailed *t* test. For multiple comparisons, analysis of variance (ANOVA) was used with Fisher's test for post hoc comparisons. Differences were considered statistically significant if $p < .05$.

Results

GH Gene Expression, IR-GH Reserve, and IR-GH Secretion in Pituitary and Cell Extracts With Aging

To understand the mechanism of age-related decline in GH secretion, GH mRNA levels and IR-GH content were analyzed in pituitary extracts from rats of different ages. As shown in Figure 1A, pituitary GH mRNA accumulation, quantified by Northern blot, was markedly decreased in the 24-month rats (24m vs 3m 42.5 ± 7.3%; $p < .001$). Pituitary IR-GH content, which is equivalent to pituitary GH reserve, showed a greater decrease in the 24-month group (24m vs 3m 7.9 ± 5.5%; $p < .001$) (Figure 1B). These results indicate that the potential mechanism of this alteration may be of transcriptional origin with the subsequent effect on GH secretion.

To determine the behavior of the somatotropes from young and old rats in our in vitro system, IR-GH release and GH mRNA levels were analyzed in pituitary cells from old and young rats from 4 to 72 hours. After the stabilizing period, media were replaced and the cells were incubated for another 72 hours. As shown in Figure 1C and D, GH mRNA levels, quantified by Northern blot, were markedly decreased in the 24-month rat cells after 4 and 72 hours in culture (24m vs 3m 48.5 ± 5.8% and 43.8 ± 3.5, Figure 1C and D, respectively; $p < .001$). A significantly reduced basal GH secretion (Figure 1E) was observed in cells from 24-month rats compared with cells from 3-month rats after 4 hours in culture ($p < .001$); this reduced secretion was maintained for the 72 hours of culture. These data indicate that, in our in vitro system, somatotropes from both groups maintain the characteristics observed in aged animals: a significant decrease in GH secretion and GH mRNA levels, and a drastic decreased IR-GH content (data not shown).

Pituitary Pit-1 Gene Expression in Aging Rats

The decline in GH gene expression with age is a constitutive pituitary alteration that might result from a parallel modification of some transcription factors specifically involved in pituitary GH gene expression. As pituitary Pit-1 has been shown to bind to and activate the GH promoter, its expression in the pituitary of old compared with young rats was studied.

As depicted in Figure 2A, the presence of approximately 3.1 and 1.2 Kb Pit-1 mRNA transcripts was detected in the anterior pituitaries of 3-month and 24-month rats. In pituitaries from 24-month rats, the intensity of the 3.1 Kb lane decreased when compared with that of the 3-month rats (24m vs 3m 77 ± 5.5%; $p < .05$), whereas the 1.2 Kb lane did not change with age. The densitometric analysis of both mRNA lanes together did not confirm a significant alteration of Pit-1 expression with advancing age. Furthermore, pituitary Pit-1 protein content quantified by Western blot remained unchanged (Figure 2B). Therefore, no significant differences in Pit-1 expression, measured either by mRNA accumulation or protein content, were observed between young and old rats.

Pituitary CREB Gene Expression With Aging

The GHRH transcriptional control of GH gene expression seems to be mediated, at least in part, by the transcription factor CREB. When 24-month and 3-month rats were compared, no significant difference was observed in the pituitary accumulation of CREB mRNA (Figure 3A); neither was there any difference in CREB protein content or total and phosphorylated forms (Figure 3B). These results indicate that

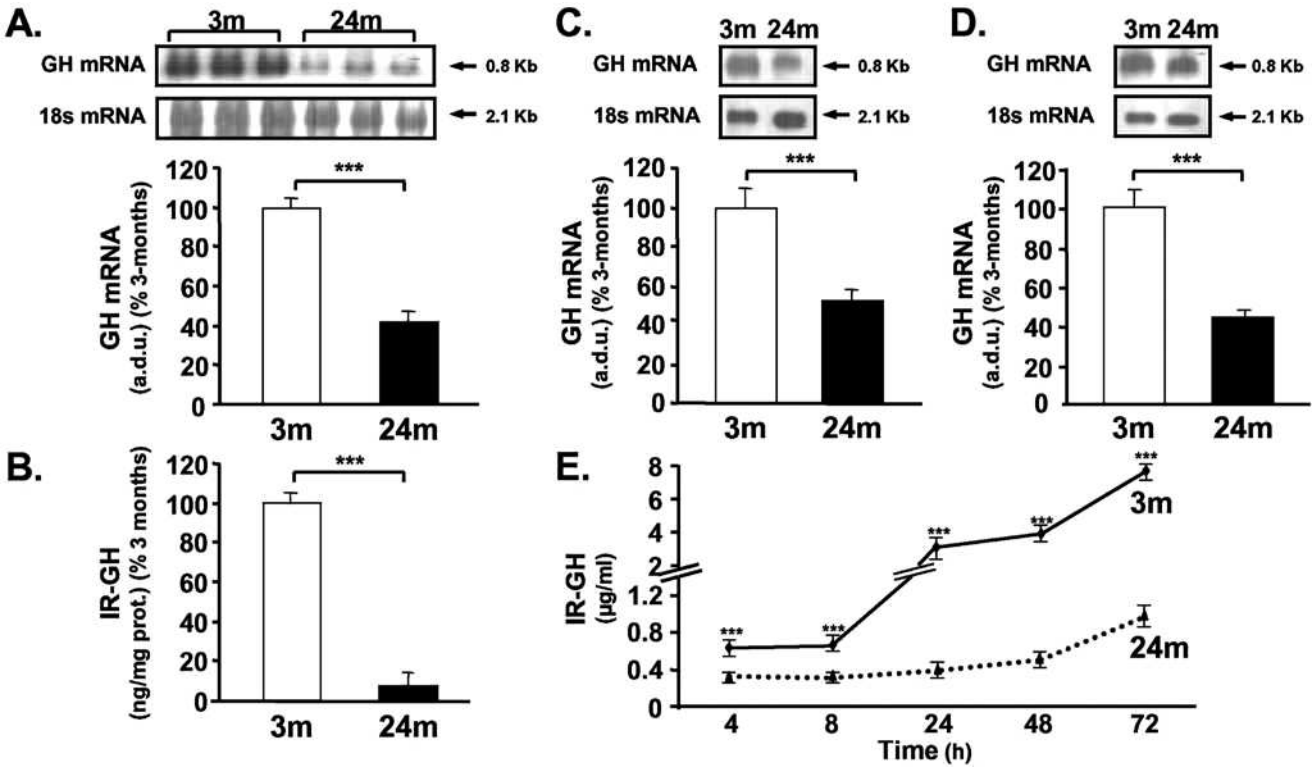

Figure 1. Pituitary growth hormone (GH) messenger RNA (mRNA) levels, immunoreactive rat (IR)-GH content and secretion, and GH mRNA levels in somatotropes from young (3-month [3m]) and old (24m) rats in culture. **A,** Pituitary GH mRNA levels were measured by Northern blot. *Upper panel:* representative Northern blot. *Lower panel:* quantification of GH mRNA bands by scanning densitometry. Two micrograms of total pituitary RNA was subjected to Northern blot using GH probe. After correction for 18s levels, optical density units were adjusted so that the ratio obtained from pituitaries of 3m rats equaled 100. **B,** IR-GH content was measured by radioimmunoassay (RIA). Values were adjusted so that the values obtained from pituitaries of 3m rats equaled 100. **C** and **D,** GH mRNA levels were measured by Northern blot. Pituitary cells from the two groups were cultured for 4 (C) or 72 hours (D). *Upper panel:* representative Northern blot. *Lower panel:* quantification of GH mRNA bands by scanning densitometry. Northern blot and data evaluation were performed as in A. **E,** Somatotrope IR-GH secretion. Pituitary cells from young and old rats were cultured for 72 hours. Aliquots of medium were obtained at 4, 8, 24, 48, and 72 hours to measure IR-GH secretion by RIA. Values represent mean ± standard error ($n = 12$). ***$p < .001$ vs 3m group. a.d.u., arbitrary densitometric units.

in aging rats there is no alteration of the pituitary expression of this transcription factor that could account for the GH gene expression decay that occurs in aging.

Pituitary GHRH-R Gene Expression and Sensitivity of Pituitary Cells to GHRH in Aging

The action of GHRH on GH gene expression and secretion is mediated by the GHRH-R. Alterations of this receptor may be relevant to understand the mechanism of the gradual decay of GH in aging. Therefore, GHRH-R mRNA levels in pituitary extracts from young and old rats were analyzed. As shown in Figure 4A, the presence of 2.5 and 4 Kb GHRH-R mRNA transcripts was detected in the anterior pituitaries of 3-month and 24-month rats. The relative abundance of the two transcripts was significantly diminished in pituitaries from 24-month rats compared with those of 3-month rats (24m vs 3m: 23.6.7 ± 6.7%, $p < .001$ and 42.1 ± 3.8%, $p < .001$ of 4 and 2.5 Kb transcripts, respectively). This significant decrease of GHRH-R gene expression suggests that this signaling pathway might have an important role in driving the decline over age of GH secretion.

To determine the functional implication of the lower GHRH-R mRNA content in pituitary cells from old rats, we analyzed the capacity of GHRH to release GH from cultured anterior pituitary cells from old and young rats, and the ability of somatotropes to release GH upon a receptor-independent stimulant of adenylyl cyclase such as FK. Primary cultures of anterior pituitary cells from 3-month and 24-month rats were treated with GHRH or FK for 4 hours. As shown in Figure 4B, a reduced basal GH secretion was maintained in cells from 24-month rats compared with cells from 3-month rats ($p < .01$). The exposure to GHRH or FK produced a significant increase of GH secretion in the cells from the two groups. In the cells from 24-month rats, the levels of IR-GH after stimulation by both secretagogues were equal or even greater than the basal values of cells from 3-month rats. When the IR-GH secretory response was calculated after correction for the basal absolute values and expressed as fold increase over their corresponding basal secretion, there was not any significant difference in the response between the two groups (GHRH: 3m, 2.68 ± 0.19; 24m, 2.61 ± 0.09 and FK: 3m, 2.87 ± 0.24; 24m, 3.01 ± 0.18) (Figure 4C). These results suggest that the pituitary cells from old rats maintain the same capacity as the cells from young rats to respond to exogenous GHRH, and that the lower absolute response of the old rats is brought about by their lower GH content reserve.

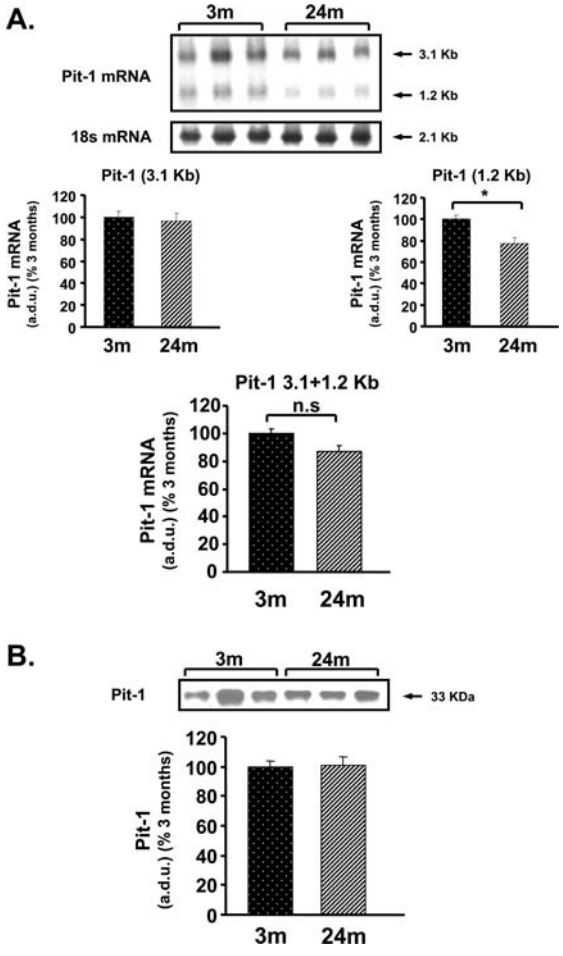

Figure 2. Pituitary Pit-1 messenger RNA (mRNA) levels and Pit-1 protein in young (3 month [3m]) and old (24m) rats. **A,** Pit-1 mRNA levels were measured by Northern blot. *Upper panel:* representative Northern blot. *Middle* and *lower panels:* quantification of 3.1- and 1.2 Kb Pit-1 transcript bands by scanning densitometry. Fifteen micrograms of total pituitary RNA was subjected to Northern blot using Pit-1 probe. After correction for 18s levels, optical density units were adjusted so that the ratio obtained from pituitaries of 3m rats equaled 100. **B,** Pit-1 protein was measured by Western immunoblot. *Upper panel:* representative Western blot. *Lower panel:* quantification of Pit-1 by scanning densitometry. Ten micrograms of protein from pituitary extracts were subjected to Western immunoblot. Optical density units were adjusted so that the values obtained from pituitaries of 3m rats equaled 100. Values represent mean ± standard error ($n = 12$). *$p < .05$. n.s., not significant vs 3m group; a.d.u., arbitrary densitometric units.

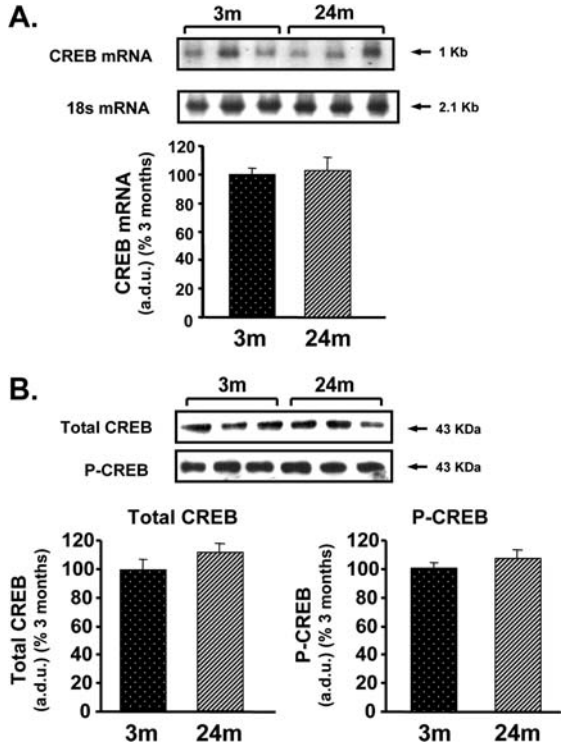

Figure 3. Pituitary cAMP-response element-binding protein (CREB) messenger RNA (mRNA) levels and CREB protein in young (3 month [3m]) and old (24m) rats. **A,** CREB mRNA levels were measured by Northern blot. *Upper panel:* representative Northern blot. *Lower panel:* quantification of CREB mRNA bands by scanning densitometry. Fifteen micrograms of total pituitary RNA was subjected to Northern blot using CREB probe. After correction for 18s levels, optical density units were adjusted so that the ratio obtained from pituitaries of 3m rats equaled 100. **B,** CREB and phospho-CREB (P-CREB) proteins were measured by Western immunoblot. *Upper panel:* representative Western blot of total CREB. *Middle panel*: representative Western immunoblot of P-CREB. *Lower panel:* quantification of total CREB and P-CREB by scanning densitometry. Ten micrograms of protein from pituitary extracts were subjected to Western immunoblot. Optical density units were adjusted so that the values obtained from pituitaries of 3m rats equaled 100. Values represent mean ± standard error ($n = 12$). a.d.u., arbitrary densitometric units.

Pituitary GHS-R Gene Expression and Sensitivity of Pituitary Cells to Ghrelin With Increasing Age

Given that ghrelin plays an important role in the physiological regulation of GH by acting also at the pituitary level, GHS-R mRNA levels were quantified by real time RT-PCR in pituitary extracts from rats of different ages. A significant decrease in the levels of GHS-R mRNA was observed in 24-month rats (24m vs 3m 36.8 ± 7.4%; $p < .01$) (Figure 5A). These data indicate an alteration of pituitary GHS-R gene expression that might contribute to a decrease in pituitary ghrelin efficacy and/or intensity of signalling with age.

To assess this possibility, cultured anterior pituitary cells from young and old rats were treated with ghrelin for 4 hours. As described previously, a reduced basal GH secretion was observed in cells from 24-month rats, compared with cells from 3-month rats ($p < .001$) (Figure 5B). The exposure to ghrelin produced a significant increase of GH secretion in the cells from the two groups. When the IR-GH secretory response was calculated after correction for the basal absolute values and expressed as fold increase over their corresponding basal secretion, there was no significant difference in the response (3m: 1.54 ± 0.03; 24m: 1.48 ± 0.05) (Figure 5C). These data indicate that pituitary cells from old rats maintain the same capacity to respond to exogenous ghrelin as the cells from young rats.

Pituitary sstr2 and sstr5 Gene Expression and Sensitivity of Pituitary Cells to SS With Increasing Age

SS is the major inhibitory signal of GH secretion; therefore, mRNA levels of sstr2 and sstr5 subtypes, the most highly expressed in the pituitary and functionally

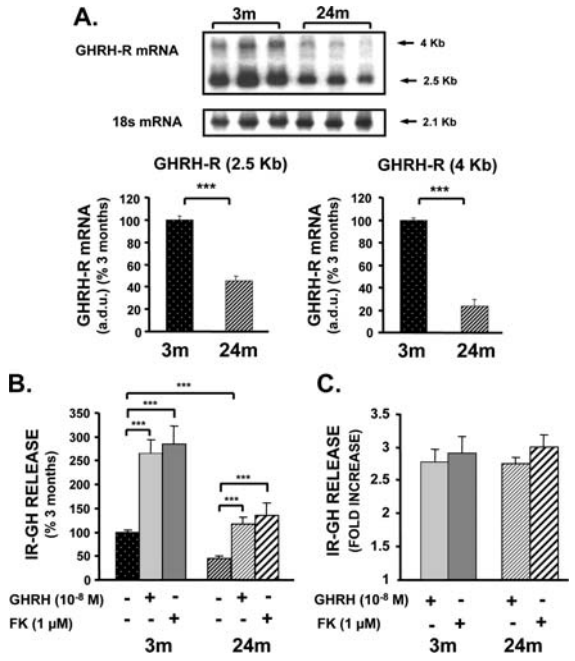

Figure 4. Pituitary growth hormone releasing hormone receptor (GHRH-R) messenger RNA (mRNA) levels and sensitivity of pituitary cells to GHRH in relation with age. **A,** Pituitary GHRH-R mRNA levels were measured by Northern blot. *Upper panel:* representative Northern blot. *Lower panel:* quantification of GHRH-R mRNA bands by scanning densitometry. Fifteen micrograms of total pituitary RNA from young (3 month [3m]) and old (24m) rats were subjected to Northern blot using GHRH-R probe. After correction for 18s levels, optical density units were adjusted so that the ratio obtained from pituitaries of 3m rats equaled 100. Values represent mean ± standard error (*SE*) ($n = 12$). ***$p < .001$ vs 3m group. a.d.u., arbitrary densitometric units. **B,** Response of pituitary cells to GHRH. Pituitary cells of young and old rats were incubated in the absence or presence of GHRH (10^{-8} M) or forskolin (FK; 1 μM) for 4 hours. Figure represents immunoreactive rat (IR)-GH in culture medium. Data were adjusted so that values obtained in untreated pituitary cells from 3m rats equaled 100. **C,** Results are expressed as fold increase of the IR-GH response to GHRH or FK after correction for the basal absolute values. Values represent mean ± *SE* of four experiments ($n = 16$). ***$p < .001$ vs their respective age control group.

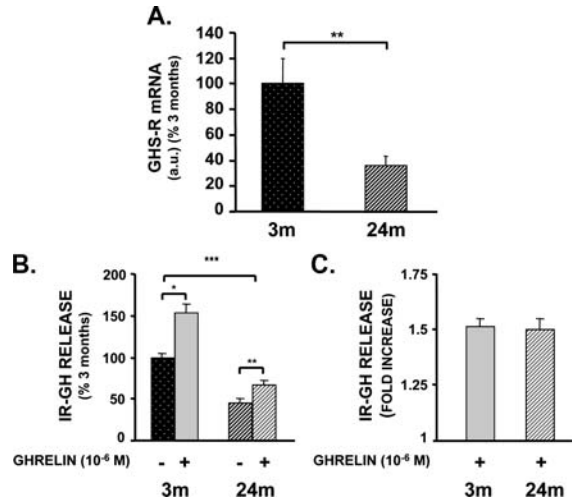

Figure 5. Pituitary growth hormone secretagogue receptor (GHS-R) messenger RNA (mRNA) levels and sensitivity of pituitary cells to ghrelin in relation with age. **A,** GHS-R mRNA levels were measured by real time polymerase chain reaction (PCR). Two micrograms of total pituitary RNA from young (3 month [3m]) and old (24m) rats was reverse transcribed, amplified, and quantified by real time PCR as described in Methods. After correction for GAPDH levels, the data were adjusted so that the values obtained from pituitaries of 3m rats equaled 100. Values represent mean ± *SE* ($n = 12$). **$p < .01$ vs 3m group. a.u., arbitrary units. **B,** Response of pituitary cells to ghrelin. Pituitary cells of young and old rats were incubated in the absence or presence of ghrelin (10^{-6} M) for 4 hours. Figure represents immunoreactive rat (IR)-GH in culture medium. Data were adjusted so that the values obtained in untreated pituitary cells from 3m rats equaled 100. **C,** Results are expressed as fold increase of the IR-GH response to ghrelin after correction for the basal absolute values. Values represent mean ± *SE* of four experiments ($n = 16$). ***$p < .001$ vs their respective age control group.

active of SS receptors, were analyzed by real time RT-PCR in pituitary extracts from rats of different ages. As shown in Figure 6A and B, a significant decrease in the levels of sstr2 and sstr5 was observed in 24-month rats compared with 3-month rats (24m vs 3m: 61.58 ± 5.7% Figure 6A and 58.67 ± 4.3% Figure 6B, respectively; $p < .01$).

It has been postulated that a higher pituitary sensitivity to the SS signal may be the mechanism involved in the decrease of GH secretion in aging. Because we found a lower expression of SS receptor in pituitary of old rats, we studied the pituitary sensitivity to SS in relation to age. To this end, cultured anterior pituitary cells from 3-month and 24-month rats were treated with SS for 4 hours. The exposure to SS produced a significant decrease of GH secretion in the cells from the two groups (Figure 6C). When the SS effect on IR-GH secretion was calculated after correction for the basal absolute values and expressed as a percentage of their corresponding basal secretion, there was no significant difference between the 3-month and 24-month rats (3m: 45.5 ± 3.8%; 24m: 59.7 ± 8% vs their own controls) (Figure 6D).

To test for the inhibitory effect of SS on GHRH- and ghrelin-induced GH secretion, cultured anterior pituitary cells from 3-month and 24-month rats were treated with GHRH or ghrelin alone or plus SS for 4 hours. As described above, the exposure of pituitary cells to GHRH and ghrelin significantly increased GH secretion in the cells of both groups (Figure 6E and F). The addition of SS partially inhibited GHRH-induced GH secretion in both groups (3m: 50%; 24m: 41% vs each corresponding GHRH-treated group) and reduced the ghrelin-induced GH secretion below the basal GH values in both groups (3m: 47%; 24m 41% vs each corresponding ghrelin-treated group). A tendency to lower sensitivity to SS, both on basal IR-GH secretion and on GHRH- and ghrelin-induced IR-GH secretion was observed in the cells from old rats. These results suggest that an increased sensitivity to SS does not appear to be responsible for the gradual decline of GH secretion with advancing age.

Pituitary IGF-I-R Gene Expression and Sensitivity of Pituitary Cells to IGF-I in Aging

To study the potential implication of IGF-I in the decline of GH with aging, pituitary IGF-I-R and protein content were analyzed by RNAse protection assay and Western blot,

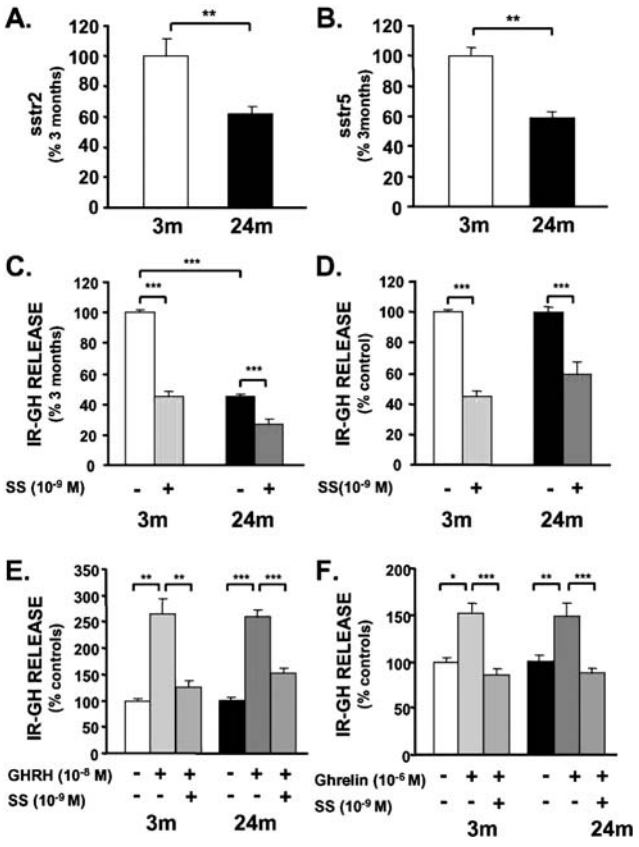

Figure 6. Pituitary somatostatin (SS) receptor subtypes messenger RNA (mRNA) levels and sensitivity of pituitary cells to SS in young (3 month [3m]) and old (24m) rats. Pituitary sstr2 (**A**) and sstr5 (**B**) mRNA levels were measured by real time polymerase chain reaction (PCR). Two micrograms of total pituitary RNA was reverse transcribed, amplified, and quantified by real time PCR as described in Methods. After correction for GAPDH levels, the data were adjusted so that the values obtained from pituitaries of 3m rats equaled 100. Values represent mean ± standard error (*SE*) ($n = 12$). $^{**}p < .01$ vs 3m group. a.u., arbitrary units. **C,** Response of pituitary cells to SS. Pituitary cells of young and old rats were incubated in the absence or presence of SS (10^{-9} M) for 4 hours. Figure represents immunoreactive rat (IR)-GH in culture medium. Data were adjusted so that values obtained in untreated pituitary cells from 3m rats equaled 100. **D,** Results are expressed as percentage of their respective age control group. **E,** Effect of SS on growth hormone releasing hormone (GHRH)-induced GH secretion. Pituitary cells of young and old rats were incubated in the absence or presence of GHRH (10^{-8} M) or GHRH+SS (10^{-9} M). Figure represents IR-GH in culture medium. Results are expressed as percentage of their respective age control group after correction for the basal absolute values. **F,** Effect of SS on ghrelin-induced GH secretion. Pituitary cells of young and old rats were incubated in the absence or presence of ghrelin (10^{-6} M) or ghrelin+SS (10^{-9} M). Figure represents IR-GH in culture medium. Results are expressed as percentage of their respective age control group after correction for the basal absolute values. Values represent mean ± *SE* of four experiments ($n = 16$). $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$ vs their respective age control group.

respectively. As shown in Figure 7A and B, both IGF-I-R mRNA and protein levels were similar in young and old rats.

It has been postulated that a higher pituitary sensitivity to IGF-I may account for the decrease of GH secretion in aging. To clarify this point, we studied the pituitary sensitivity to IGF-I in relation to age. To this end, cultured anterior pituitary cells from 3-month and 24-month rats were treated with IGF-I for 72 hours. The exposure to IGF-I produced a significant decrease of GH mRNA levels and IR-GH secretion in the cells from the two groups (Figure 7C and E). When this effect was expressed as a percentage of their corresponding basal values, there was no significant difference in the inhibitory effect of IGF-I on GH mRNA and IR-GH secretion between the 3-month and 24-month rats (GH mRNA levels: 3m, 41 ± 8.9%; 24m, 52 ± 18% and IR-GH: 3m, 44 ± 17%; 24m, 61 ± 10.2% vs their own controls) (Figure 7D and F). There was a trend for reduced sensitivity to IGF-I in the cells from old rats. These data indicate that an increased sensitivity to IGF-I is not involved in the mechanism of the GH gene expression and GH secretion decline with advancing age.

Somatotrope Population in Aging Pituitaries

To answer the traditional question of a potential age-related alteration in the relative proportion of differentiated pituitary cells, cytometric studies were done.

The dissociation procedure yielded about 2.8×10^6 cells per anterior pituitary gland in 3-month rats and 5.7×10^6 cells per anterior pituitary gland in 24-month rats (24m vs 3m, 203 ± 12.3%; $p < .001$). Cell viability, as measured by 7-AAD exclusion test, was >90% in both 3-month and 24-month cells, with no significant difference. There was no change in the percentage of pituitary cells immunoreactive to GH with aging, quantified by flow cytometry (Figure 8C). These data together suggest an increase in the total number of cells together with a relative increase of GH secretory cells in the pituitaries from old rats.

Discussion

Reduction of the GH/IGF-I axis activity is a hallmark of the hormonal changes with aging. The GH/IGF-I decrease in senescence has important clinical implications, particularly as GH deficiency might contribute to the gradual decline in tissue functions associated with normal aging (1). The main goals of the present studies were to elucidate whether the decrease in GH secretion during aging is a pituitary-constitutive alteration and to determine the potential reversibility of the GH secretion decay by analyzing the pituitary capacity to respond to different stimuli. The key findings of the present study include the demonstration that aging does not modify Pit-1 and CREB expression and does not cause a decrease in the somatotrope population. Moreover, the adenylyl cyclase–cAMP pathway remains unaltered in the old somatotropes. Additionally, we show that the sensitivity of somatotropes to ghrelin, to the inhibition by SS of GHRH- and ghrelin-induced GH and to IGF-I is not modified by aging.

In this study, we confirm our previous findings (8,9,41), which showed that GH gene expression, pituitary IR-GH content, and GH secretion diminish with age. The decrease in GH secretion rate, demonstrated by the decrease in GH amplitude or frequency of pulses (7), appears to be dependent on the lower pituitary reserve due to a decrease in GH gene expression, indicating that a transcriptional mechanism might account for the decline in GH secretion that occurs with aging. The concept that GH is regulated by different developmental stages (such as puberty and aging)

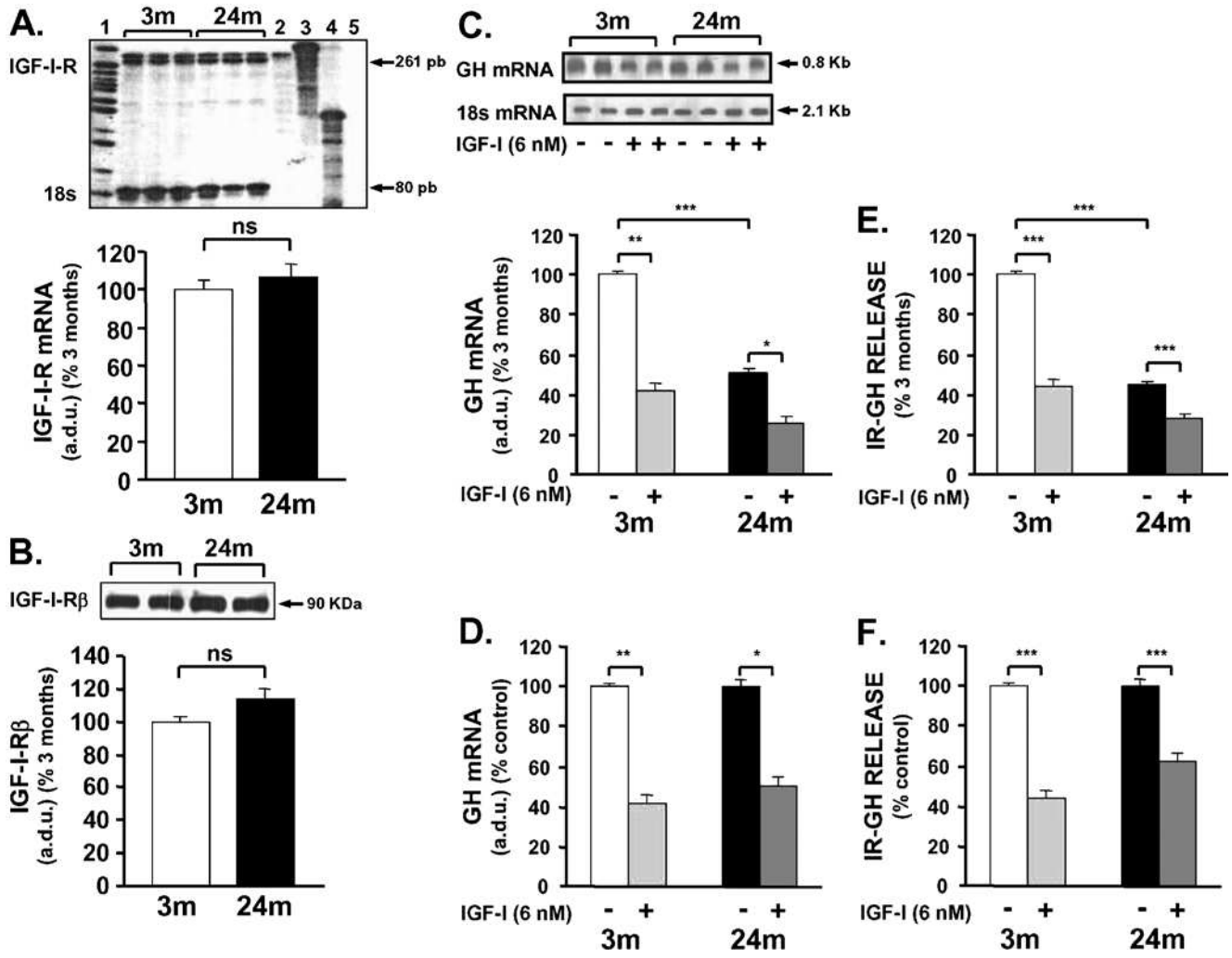

Figure 7. Pituitary insulin-like growth factor I (IGF-I) receptor messenger RNA (mRNA) levels and IFG-I receptor (IGF-I-R)β protein and sensitivity of pituitary cells to IGF-I in young (3 month [3m]) and old (24m) rats. **A,** IGF-I-R mRNA levels were measured by RNase protection assay. *Upper panel:* representative gel. *Lower panel:* quantification of IGF-I-R mRNA protected fragment by scanning densitometry. Ten micrograms of total pituitary RNA was subjected to solution hybridization/RNase protection assay using the antisense IGF-I-R and 18S probes. The positions of each protected fragment are indicated on the *right*. *Lane 1:* molecular-weight marker. *Lane 2:* transfer ribonucleic acid (tRNA) control. *Lane 3:* undigested IGF-I-R probe. *Lane 4:* undigested 18S probe. *Lane 5:* IGF-I-R and 18S probes after RNase A and T1 digestion. **B,** IGF-I-Rβ protein was measured by Western immunoblot. *Upper panel:* representative Western blot. *Lower panel:* quantification of IGF-I-Rβ by scanning densitometry. Ten micrograms of protein from pituitary extracts was subjected to Western blot. Optical density units were adjusted so that the values obtained from pituitaries of 3m rats equaled 100. Values represent mean ± standard error (*SE*) ($n = 12$). n.s., not significant vs 3m group; a.d.u., arbitrary densitometric units. **C,** Effect of IGF-I on growth hormone (GH) mRNA levels in pituitary cells measured by Northern blot. Pituitary cells of young and old rats were incubated in the absence or presence of IGF-I (6 nM) for 72 hours. *Upper panel:* representative Northern blot. *Lower panel*: quantification of GH mRNA bands by scanning densitometry. Two micrograms of total cellular RNA was subjected to Northern blot using GH probe. After correction for 18s levels, optical density units were adjusted so that the ratio obtained from 3m rats equaled 100. **D,** Results are expressed as percentage of their respective age control group after correction for the basal absolute values. **E,** Effect of IGF-I on immunoreactive rat (IR)-GH secretion. Pituitary cells of young and old rats were incubated in the absence or presence of IGF-I (6 nM) for 72 hours. Figure represents IR-GH in culture medium. Data were adjusted so that values obtained in untreated pituitary cells from 3m rats equaled 100. **F,** Results are expressed as percentage of their respective age control group after correction for the basal absolute values. Values represent mean ± *SE* ($n = 16$). $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$ vs their respective age control group. a.d.u., arbitrary densitometric units.

in animals and humans is strongly supported by our results. Thus, puberty could be a model of GH gene overexpression and secretion, and aging a profile of relative or partial GH deficiency.

We also found that after 6 days in vitro the cumulative GH secretion and GH mRNA levels are lower in pituitary cells from old rats, which indicates that in our in vitro system the cultured somatotropes retain the characteristics observed in vivo.

Pituitary-specific expression of the GH gene is Pit-1 dependent (23). Given that Pit-1 and GH promoters are stimulated by cAMP, we studied the potential alteration with age of the pituitary transcription factors Pit-1 and CREB, both of which are involved in GH gene expression. Our results indicate that the pituitary expression of the transcription factors Pit-1 and CREB in old rats is not significantly different from that in young rats. Similar Pit-1 results were obtained in Sprague-Dawley rats by Korytko

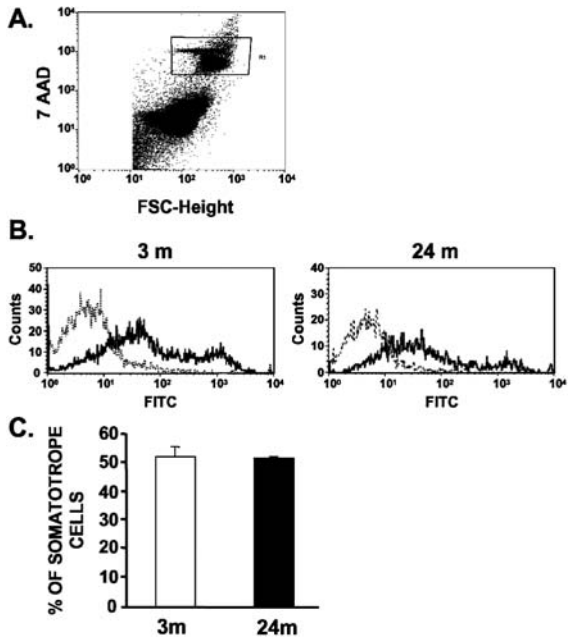

Figure 8. Growth hormone-labeled cells in the pituitaries from young (3 month [3m]) and old (24m) rats. **A,** Distribution of pituitary cells on 7-amino actinomycin/forward scatter (7-AAD/FSC) diagram used to select nucleated cells. **B,** Representative fluorescein isothiocyanate (FITC) fluorescence histogram of pituitary cells from 3m and 24m rats. *Dashed line:* control; *black line:* GH-expressing cells. **C,** Percentage of immunoreactive GH-positive cells relative to the total number of cells. Values represent mean ± standard error ($n = 8$).

and colleagues (42), who found no difference in Pit-1 mRNA levels when analyzing pituitaries from 70-day and 12-month rats. In 18-month rats of the same strain, Gaudreau (43) observed no change in Pit-1 mRNA or protein compared with younger rats. Similar findings have been shown in rats with streptozotocin-induced diabetes, where the decrease in GH gene expression is not related to changes of Pit-1 expression (44). However a decrease of Pit-1 gene expression in old female mice has been described in a recent study (45). There are not positive data showing that CREB might be involved in this mechanism which is in agreement with the results found in this study. These data strongly suggest that changes in the expression of these transcription factors are unlikely to contribute to the gradual downfall of GH secretion with advancing age.

We next studied whether the specific receptors of the three major regulatory signals of GH secretion—growth hormone-releasing hormone (GHRH), ghrelin, and somatostatin (SS)—might be involved in the decline of GH in aging. We found that a significant decrease in the pituitary levels of GHRH-R, GHS-R, sstr2, and sstr5 mRNAs occurs in aging. Our results confirm previous studies with respect to GHRH-R. In fact, a progressive decrease of GHRH-R levels in rats of 6 and 24 months of age has been described (46). In another study of 18-month rats, a decrease of the GHRH-R 2.5 Kb transcript that exhibits high-affinity binding and functionality (47), and an increase of the 4 Kb transcript but with a decrease in the total GHRH-R mRNA was found in the old rats compared with young rats (48). As to pituitary age-related GHS-R gene expression, there is only one study in old female mice that confirms our finding of lower GHS-R mRNA in older rats. In the same study, a diminution of the sstr2 and sstr5 mRNA levels in the pituitary of older mice is in agreement with our results in the rat (45). A more recent study, in rats of 16-months, confirmed a decrease of sstr2 and sstr5 mRNA levels compared with younger rats (46).

Therefore, an age-related decline in the pituitary gene expression of the receptors involved in the regulation of GH gene expression and secretion, including those related with stimulatory effects (such as GHRH and ghrelin) and with inhibitory action (such as SS), is clearly established. This age-related perturbation could be a primary event but most probably is caused by a drop of hypothalamic GHRH (49), as the expression of GHRH-R, GHS-R, and sstr2 is stimulated by this signal. In an in vitro study in adult male rats, a critical effect of GHRH on GHRH-R mRNA accumulation was found to be related to the duration of GHRH exposure; thus, 72-hour exposure stimulated GHRH-R mRNA over the control values (50). Another recent study in ovine somatotropes has shown that GHRH treatment in vitro increased the levels of GHRH-R and GHS-R mRNA (51). This hypothesis is also supported by other studies which showed that in spontaneous dwarf rats (SDR), in which a mutation of the GH gene results in total absence of the hormone, treatment with GHRH stimulated GHRH-R, GHS-R, and sstr2 gene expression (52) but inhibited sstr5. However, in normal pituitary cells, when exposed for 4 hours to GHRH, GHRH-R and sstr5 mRNA levels decreased, whereas those of GHS-R and sstr2 increased. This opposite effect of GHRH on the regulation of SS receptor has been confirmed in another study that showed that GHRH administration increased sstr2 mRNA and decreased sstr5 mRNA levels in rat pituitaries (53). As the decrease of GHRH would not seem to explain the diminished sstr5 expression, other still unknown mechanisms might be implicated. Some authors support the hypothesis that hypothalamic SS upregulates pituitary SS-receptor subtype levels (54,55). These early reports, carried out in pituitary cell lines, showed that after 24 and 48 hours SS can increase sstr levels, but the response of sstr2 to SS is mainly biphasic and relative to the time of exposure to the peptide. A more recent report on cultured rat pituitary cells demonstrated that SS increases sstr5 and has no effect on sstr2 (56). In vivo studies have shown that the immunoneutralization of endogenous SS does not alter basal or GHRH-mediated SS-receptor subtype expression (53,57). The discrepancy between these data may be due to the fact that the in vitro studies examined the effect of exogenous SS stimulation, whereas the in vivo studies examined the effect of the suppression of endogenous SS; there could have been compensatory mechanisms in these in vivo studies. Therefore, if SS really upregulated sstr5, the drop in pituitary sstr5 with advancing age may reflect the decrease of hypothalamic SS. These findings suggest that the decline of the GH/IGF-I system with age is not a constitutive primary pituitary alteration and open up the possibility that the GH pituitary alterations of aging might be reversible. Our data and those from other authors have

shown that somatotropes comprise approximately half of young and old pituitary cells in humans and rats (58,59). Within the pituitary, GHS-R, sstr2, and sstr5 are expressed in somatotropes together with other pituitary cells (60,61). The age-related changes in pituitary GHS-R, sstr2 and sstr5 most likely reflect changes that occur in the somatotropes, although other pituitary cells may also play a role.

At present, the in vivo and in vitro sensitivity of old pituitary cells to GHRH is a matter of controversy, and there are not well-established data in either aging humans or aging rats. The in vivo response to GHRH has been described either as a diminution with age both in humans (62) and in rats (30) or as a response of the same magnitude in aging men (31,63) and rats (64). In in vitro studies, a diminution (65) or no change (30,33) in the somatotrope responsiveness to GHRH with age has been found. The in vivo studies of sensitivity to GHS with age showed a lower GH response to GHS with age (32). It is difficult to assess a direct pituitary action of GHS on GH release in in vivo studies because of the releasing activity of GHS on hypothalamic GHRH. There are no reliable in vitro studies concerning this issue. Our data indicate that the relative response of IR-GH to GHRH, FK, and ghrelin was not significantly different between young and old pituitary cells when compared with their own basal secretion and expressed as fold increase. Furthermore, in cells from old rats, the levels of IR-GH secreted after a pharmacological stimulus with high doses of GHRH and FK were similar to those found in the unstimulated cells from young rats. Therefore, old somatotropes maintain the normal physiological capacity to respond to secretagogues, although the higher GH reserve of young cells determines a greater absolute IR-GH response. These data indicate that the lower GH secretion that occurs with aging could be reversed by GHRH, as has been reported, and support that secretagogues are an alternative treatment for GH deficiency in elderly people.

Earlier studies, carried out in vivo or in superfused pituitary slices, indicated that the sensitivity of old somatotropes to SS was increased (33,66). Our results, obtained in different experimental conditions, indicate that SS inhibits basal and GHRH- and GHS-induced GH release with similar intensity in young and adult pituitary cells. Thus, our results suggest that the sensitivity of pituitary cells to SS does not increase with age, which is in agreement with other authors who observed that SS equally inhibits the GH release in pituitary cells or fragments of old and young rats (67,68). These results, together with the decrease of sstr2 and sstr5 expression (the two SS receptor types most functional in the pituitary) and the decrease of SS in the median eminence (29) and in hypothalamic extracts (7,69), question the hypothesis of an increased somatostatinergic tone as the mechanism of the GH expression and secretion waning that occurs in aging. There are also reports showing increased SS levels in the hypothalamus of aged rats (70) or supporting an age-related increase in the efficiency of translation of SS mRNA (71).

Our study on IGF-I, another inhibitory factor of GH secretion, does not support that IGF-I plays a role in the mechanism of GH decline in old rats. We observed no alteration of IGF-I-R mRNA levels and IGF-I-R protein content in the pituitaries of aging rats. Our findings agree with data from food-deprived rats, in which the decrease in GH and IGF-I serum levels is not accompanied with parallel pituitary changes in IGF-I-R mRNA (72). In addition, our results indicate that the sensitivity of pituitary cells to IGF-I does not increase with age. Chapman and colleagues (37) obtained similar results in a study of elderly persons in whom they blocked the fasting-induced GH with exogenous IGF-I, and observed that the IGF-I treatment decreased GH serum levels to a lesser extent in aged men than in young men, despite the fact that IGF-I serum levels were greater in the older than the younger men. This in vivo study was unable to establish whether the IGF-I feedback occurs at the pituitary or hypothalamic level. Our study, which analyzes the direct effect of IGF-I on somatotropes in aging, together with the well-known decline of circulating IGF-I, clearly rules out that changes in IGF-I action at the pituitary level may account for the decline of GH in aging.

Although it has been demonstrated that the pituitary of old rats is heavier than that of young rats, a reduction in the population of pituitary somatotropes has been postulated as a mechanism involved in the diminution of GH secretion of aging. Our results confirm an increase in the total cell number per pituitary in aging rats as shown in previous studies in pituitaries dissociated from 26-month rats (73) and 20-month rats (67). We have also observed that the percentage of GH immunoreactive cells was similar in the pituitaries from young and old rats. Other studies in old pituitaries have described a decrease in the somatotrope population by immunohistochemical quantification of pituitary sections (74,75). The differences in species, rat strains, and methodologies used probably account for the variance in results among the studies. Our results strongly suggest that a diminution in the number of somatotropes is not the mechanism of the GH secretion decline of senescence and indicate that, at least in the rat, there is an increase in the total number of GH cells per pituitary with aging. This statement is also supported by the earlier results of Rossi and colleagues (76) who observed that somatotrope number increased in pituitaries of male rats of advanced age. Given that hypothalamic GHRH, which stimulates somatotrope proliferation (77), is decreased in old male rats (49), it is not clear what causes the increase in the number of somatotropes in the aging male rats.

Summary

This study demonstrates some age-related pituitary perturbations such as the decrement of pituitary IR-GH content and mRNA levels, and a lower expression of pituitary receptors: GHRH-R, GHS-R, sstr2, sstr5, and IGF-I-R. Moreover, we have found that aging does not modify Pit-1 and CREB expression in the pituitary and does not cause a decrease of somatotrope population. Additionally, we demonstrate that the capacity of GH response to pharmacological doses of secretagogues is not altered in cells from aging rats, and that the pituitary cells from old rats do not show a greater sensitivity to SS or IGF-I. Our results give experimental support to the concept of reversibility of the pituitary alteration of the GH/IGF-I

system that occurs with aging, at least with direct pituitary stimulation of endogenous GH secretion with GHRH and other secretagogues.

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